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(54) Title: SYNERGISTIC COMPOSITION AND METHODS FOR TREATING NEOPLASTIC OR CANCEROUS GROWTHS AND FOR RESTORING OR BOOSTING HEMATOPOIESIS (57) Abstract The present invention provides a synergistic composition and methods for treating neoplastic or cancerous growths as well as for treating such patients in order to restore or boost hematopoiesis. The present invention comprises administration of the combination of a cytotoxic T-lymphocyte inducing composition and at least one agent which is capable of neutralizing or down regulating the activity of tumor secreted immunosuppressive factors, separately or in combination.		

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**SYNERGISTIC COMPOSITION AND METHODS FOR
TREATING NEOPLASTIC OR CANCEROUS GROWTHS
AND FOR RESTORING OR BOOSTING HEMATOPOIESIS**

5 BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a composition and method for treating humans and animals for neoplastic or cancerous growths as well as treating such patients in order to restore or boost hematopoiesis. The composition of the present invention comprises a combination of a cytotoxic T-lymphocyte inducing composition and an agent which is capable of neutralizing or down regulating the activity of tumor secreted immunosuppressive factors.

2. Description of the Related Art

Cytotoxic T-lymphocytes (CTLs) are believed to be the major host mechanism in response to a variety of viral infections and neoplastic or cancerous growth (Greenberg et al., *Adv. Immunol.*, 49:281-355 (1991); Baxevanis et al., *Crit. Rev. Oncol.-Hematol.*, 16:157-79 (1994); Ward et al., Biological Approaches to Cancer Treatment. Biomodulation, pp. 72-97, edited by M.S. Mitchel, New York: McGraw Hill, Inc. (1993)). These cells eliminate infected or transformed cells by recognizing antigen fragments in association with various molecules (termed class I MHC molecules) on the infected or transformed cells (Baxevanis et al., *Crit. Rev. Oncol.-Hematol.*, 16:157-79 (1994); Matsumura et al., *Science*, 257:927-34 (1992); Long et al., *Immunol. Today*, 10:232-34 (1989)).

The use of soluble forms of tumor associated antigens (TAA) in subunit vaccines to stimulate tumor specific T-cell immunity is a desirable strategy for developing a safe and effective immunotherapy for cancers. The advantage of using whole protein is that after antigen processing in specialized antigen presenting cells (APC) it contains the entire repertoire of potential peptide epitopes. However, the immunization with whole soluble antigen generally does not activate CTLs. Therefore, to stimulate CTL response to specific protein antigens, various approaches focusing on improving the intracellular antigen delivery to APC have been tried. These include live viral (Moss, B., *Science*, 252: 1662-67 (1991); Takahashi et al., *PNAS USA*, 85:3105-09 (1988)) and bacterial (Aldovini et al., *Nature (London)*, 351:479-482 (1991); Sadoff et al., *Science*, 240:336-38 (1988)) vectors, non-replicating plasmid DNA inoculation (Ulmer et al., *Science*, 259:1745-49 (1993)), conjugation of protein and peptides to lipophilic compounds (Deres et al., *Nature (London)*, 342:561-64 (1989)) or ISCOM (Takahashi et al., *Nature (London)*, 344:873-75 (1990)). The major concerns for vaccines, based on viral vectors or DNA injections, are safety relating to possible DNA integration into the host cell genome which is particularly relevant to oncogenes with transforming potentials and the induction of anti-vector response *in vivo*. Furthermore, in immunocompromised individuals, it is safer to use purified antigens in combination with an appropriate non-infectious delivery system with minimal toxicity to induce an immune response.

A safe and advantageous composition by which CTL response may be induced in humans and domesticated or agriculturally important animals and includes the whole soluble protein in a non-infectious delivery system was discovered by Raychaudhuri et al. (U.S. Patent No. 5,585,103), the contents of which are hereby incorporated by reference in its entirety. The CTL inducing composition involves the use of an antigen formulation which has little or no toxicity to animals, and lacks an immunostimulating peptide (e.g., muramyl dipeptide), the presence of which would decrease the desired response. More specifically, the CTL inducing composition (PROVAX™) comprises the antigen to which the CTL response is desired and a non-toxic antigen formulation which comprises, consists or consists essentially of a stabilizing detergent, a micelle-forming agent, and a biodegradable and biocompatible oil.

However, it has been documented that tumors escape from immune surveillance by secreting factors or cytokines that exert immunosuppressive effects on the functions of both activated and precursor immune cells present locally and systemically. Therefore, cancer patients receiving therapeutic vaccines alone, vaccines which are aimed at enhancing the tumor immunity, may not fully benefit from such vaccine.

Additionally, cancer patients, especially at late stages of the disease, show suppressed hematopoietic activity due to suppression of stem and/or progenitor cells that are vital for the maintenance of healthy bone marrow. This suppression is a result of compounding factors, including radiation and chemotherapy which is used in cancer treatment as well as immunosuppressive

factors that may be upregulated by cancer treatments, such as, for example, transforming growth factor- β (TGF β), a stable family of polypeptide growth factors which are secreted by normal as well as the growing tumors of the host.

Therefore, in view of the aforementioned deficiencies attendant with
5 previously known cancer vaccines and methods of treating tumors, it should be apparent that there still exists a need in the art for more efficient immunotherapeutic treatments and compositions.

SUMMARY OF THE INVENTION

10 The inventors of the present application have surprisingly discovered that the therapeutic efficacy of a vaccine which is aimed at enhancing tumor immunity, by induction of a CTL response can be increased when such CTL inducing vaccine is used in conjunction with one or more agents which are capable of neutralizing, antagonizing, down regulating or blocking tumor-
15 secreted immunosuppressive factors, e.g., TGF β and IL-10.

Accordingly, an object of the present invention is to provide a composition comprising any adjuvant formulation capable of inducing CTL in combination with one or more agents which are capable of neutralizing, blocking, antagonizing or down regulating the activity of tumor secreted factors. A
20 particular preferred CTL inducing adjuvant comprises the CTL inducing adjuvants disclosed in U.S. Patent No. 5,585,103, issued to Raychaudhuri et al., which comprise the following: an antigen to which an antigen-specific CTL response is

to be induced agonist and a microfluidized antigen formulation, said antigen formulation comprising:

- (i) a stabilizing detergent,
- (ii) a micelle-forming agent, and
- 5 (iii) a biodegradable and biocompatible oil,

and further wherein said antigen formulation lacks an immunostimulating peptide component and is formulated as a stable oil-in-water emulsion. Preferably the agent(s) which are capable of neutralizing, blocking, antagonizing or down regulating tumor-secreted immunosuppressive factors will include anti-TGF β antibodies, transforming growth factor- β receptor fusion proteins (TGF β R-fusion
10 proteins), TGF β antagonists such as thrombospondin peptides, TGF β binding proteins and TGF β R blocking antibodies.

Another object of the present invention is to provide a method of treatment which includes the induction of a CTL response wherein the
15 improvement comprises the use of an adjuvant which induces a CTL response and an antagonist of an immunosuppressive factor, preferably TGF β , said adjuvant and antagonist can be administered sequentially or concurrently in either order.

A further object of the invention is to provide a method of treating
20 neoplastic or cancerous growths in a patient in need of such treatment.

An additional object of the present invention is to provide a method of restoring or boosting hematopoiesis in a patient.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference to the following detailed description of the preferred embodiments of the invention and to the appended claims.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents the antitumor activity of ovalalbumin/PROVAX™ and/or anti-TGFβ antibody treatment on established EG7 tumors.

Figures 2A and 2B represent the antitumor activity of E7/PROVAX™ and/or anti-TGFβ antibody treatment on HOPE2 cells.

10

Figures 3A and 3B represent the estimated level of the activated or latent forms of TGFβ-1 secreted by various cell lines after in vitro incubation in serum free medium (CHO-S SFM II, GIBCO, Cat. #91-0456) for 2 days (EL4; EG7 cells) or 5 days (3T3, KB and A431 cells) continuous culture at 37°C.

15

Figure 4 represents binding of monoclonal mouse anti-TGF-β1, β2, β3 (Genzyme Corp: Cat. # 80-1835-03) for mouse or human TGFβ present in conditioned medium obtained from either human A431 cells or murine BALB/c 3t3 cells.

20

DETAILED DESCRIPTION OF THE INVENTION

As discussed *supra*, the inventors of the present application have unexpectedly discovered that the therapeutic efficacy of a vaccine which is aimed at enhancing tumor immunity, e.g., a CTL inducing adjuvant, is increased

when it is used in conjunction with one or more agents which are capable of neutralizing or down regulating tumor secreted immunosuppressive factors. The inventors have surprisingly discovered that this combination results in synergistic enhancement of cytotoxic T lymphocyte response, thereby resulting in enhanced therapeutic response against targeted antigen-expressing cells, e.g., a tumor. Additionally, the inventors have discovered that the use of one or more agents which neutralize or down regulate the tumor secreted immunosuppressive factors in combination with the vaccine or adjuvant assists in restoring or boosting hematopoiesis.

10 The soluble inhibitory or immunosuppressive factors or cytokines which are secreted by tumor cells in order to avoid immune destruction include, for example, transforming growth factor β (TGF β) (Mukherj et al., *Curr. Opin. Oncol.*, 7:175 (1995)), interleukin 10 (IL-10) (Huber et al., *J. Immunol.*, 148:277 (1992)), prostaglandin (PGE₂) (Huang et al., *J. Immunol.*, 157:5512-20 (1996)), immunosuppressive acidic protein (IAP) (Yamaguchi et al., *Oncology*, 52:1-6 (1995)) and Lipocortin-1 (LC1) (Koseki et al., *Surg. Today*, 27:30-39 (1997)). TGF β has been shown as a tumor associated immunosuppressive molecule from studies done in the glioblastoma (Brooks et al., *J. Exp. Medicine*, 136:1631-47 (1972)). Ample evidence indicates that

15 TGF β is produced by a variety of human cancer cells, including breast carcinoma (Knabbe et al., *Cell*, 48:417-28 (1987)), prostatic carcinoma (Ikeda et al., *Biochemistry*, 16:2406-10 (1987)), colorectal carcinoma (Coffey et al., *Cancer Res.*, 46:1164-69 (1986)), endometrial carcinoma (Boyd et al., *Cancer Res.*,

50:3394-99 (1990)) and ovarian carcinoma (Wilson et al., *P.R. Br. J. Cancer*, 63:102-08 (1991)).

TGF β was originally identified by its ability to impart a transformed phenotype to normal fibroblasts and found to be produced by virtually all the cells (Wakefield et al., *J. Cell. Biol.*, 105:965-75 (1987)). In humans, it is found in three different isoforms, TGF β 1, 2 and 3. TGF β is a pleiotropic cytokine which affects a wide range of biological activities, including immunosuppression, inflammation, hematopoiesis and wound repair (Sporn et al., *Science*, 233:532 (1986); Pallidino et al., *Ann. NY Acad. Sci.*, 593:181 (1990); Roberts et al., *Adv. Cancer Res.*, 51:107 (1988).

Of particular relevance is the potent immunosuppressive activity of TGF β (Pallidino et al., *Ann. NY Acad. Sci.*, 593:181 (1990); Roberts et al., *Adv. Cancer Res.*, 51:107 (1988); Lucas et al., *J. Immunol.*, 145:1415-22 (1990)). TGF β could exert immunosuppression by inhibiting, T and B cell proliferation (Kehrl et al., *J. Exp. Med.*, 163:1037 (1986); Kehrl et al., *J. Immunol.*, 137:3855 (1986); Kehrl et al., *J. Immunol.*, 143:1868 (1989)), LAK cell/CTL generation (Mulé et al., *Cancer Immunol. Immunother.*, 26:9 (1988); Espevik et al., *J. Immunol.*, 140:2312 (1988); Rook et al., *J. Immunol.*, 136:3916 (1986); Ranges et al., *J. Exp. Med.*, 166:991 (1987); Fontana et al., *J. Immunol.*, 143:323 (1989); Susan et al., *J. Exp. Med.*, 172:1777 (1990); Torre-Amione et al., *PNAS*, 87:1486 (1990) and function, NK cell activity (Rook et al., *J. Immunol.*, 136:3916 (1987); Susan et al., *J. Exp. Med.*, 172:1777 (1990); Torre-Amione et al., *PNAS*, 87:1486 (1990)) macrophage oxygen metabolisms

(Tsunawaki et al., *Nature*, 334:260 (1988)), IgG and IgM secretion (Kehrl et al., *J. Immunol.*, 137:3855 (1986); Kehrl et al., *J. Immunol.*, 143:1868 (1989) or by down regulating the Human Leukocyte Antigen (HLA-DR) (Czarniecki et al., *J. Immunol.*, 140:4217 (1988); Zuber et al., *Eur. J. Immunol.*, 18:1623 (1988) and IL-2R (Kehrl et al., *J. Exp. Med.*, 163:1037 (1986)).

Also of particular relevance is the affect TGF β has on hematopoiesis. TGF β has been shown to negatively regulate and even inhibit the growth of primitive hematopoietic cells (Sitnicka et al., *Blood*, 88(1):82-88 (1996); Dybedal et al., *Blood*, 86(3):949-57 (1995)). Antagonist of TGF β could, therefore, play an important role in improving established cancer therapies that are characterized by having dose-limiting myeloid suppression. Suppression is a result of compounding factors which may include both direct effects of the cancer therapeutics on hematopoiesis and indirect effects by upregulation of immunosuppressive factor. For example, Barcellos-Hoff et al., *J. Clin. Invest.*, 93:892-99 (1994) demonstrated that ionizing radiation of mice leads to a rapid increase in levels of active TGF β in mammary tissue and concomitant loss of latent TGF β .

The active form of TGF β is a 25kD homodimeric protein that is synthesized and secreted as a latent precursor form which becomes active presumably upon enzymatic cleavage (Massague et al., *Ann. Rev. Cell. Biol.*, 6:597-641 (1990)) although the exact method(s) of activation in vivo have not as yet been elucidated. There is 70% similarity found within each of the 3 major isoforms, TGF β 1, 2 and 3. Presumably, the actions of activated TGF β

are mediated via binding to various cell surface receptors. At least 3 different TGF β receptors, TGF β R-1, TGF β R-2 and TGF β R-3 have been identified (Barnard et al., *Biochim. Biophys. Acta*, 1032:79-87 (1990)). All three receptors are type I integral membrane glycoproteins and ubiquitously expressed by virtually all
5 cells in the body, except TGF β R-3 which is absent in monocytes. Both TGF β and its receptors have been cloned and expressed. Other TGF β membrane binding components have been described on fully differentiated subsets of cells and are not ubiquitously expressed. In particular endoglin (CD105), primarily expressed on endothelial and pre-B cells, has recently been shown to bind TGF β -
10 1 and β 3 isoforms (Zhang et al., *J. Immunol.*, 156:565-573 (1996))

There have been various attempts to neutralize and/or down regulate the activity of TGF β . For example, antibodies which are specific for TGF β have been suggested for use in treating tumor cells that produce TGF β to counteract the immunosuppressive effects of TGF β (Segarini et al., WO 94/09815). TGF β -
15 specific antibodies have also been found to restore or boost the growth of primitive hematopoietic cells, such as progenitor and stem cells, which were suppressed due to excess TGF β production (Dybedal et al., *Blood*, 86(3):949-57 (1995); Sitnicka et al., *Blood*, 88(1):82-88 (1996)).

A number of other strategies may be used to neutralize or down regulate
20 the active form of TGF β . For example, TGF β receptor (TGF β R) Fc-fusion proteins, especially the receptor II fusion proteins may be administered to neutralize or down regulate TGF β *in vivo*. Antibodies to TGF β receptor may block the interaction of free TGF β to the TGF β R and prevent downward

signaling events in the target cell. Also, analogs of TGF β or TGF β binding proteins, e.g., thrombospondin peptides, could compete with free TGF β for the binding to the receptor and inactivate the receptor. Further, gene therapy approaches may be utilized in order to achieve the above. Additional strategies

5 have been described to prevent activation of TGF β from its latent form which does not participate in signaling events. For example, thrombospondin peptide sequences have been described and synthesized which inhibit activation of latent TGF β (Schultz-Cherry et al., *J. Biol. Chem.*, 270:7304-7310 (1995)).

At least one agent capable of neutralizing or down regulating the

10 biological activity of tumor or host secreted immunosuppressive factors is present in a therapeutically effective amount. In a preferred embodiment the agent is present in an amount ranging from about 5 to about 1000 mg per square meter.

The CTL inducing composition involves the use of an antigen formulation

15 which has little or no toxicity to animals, and lacks an immunostimulating peptide (e.g., muramyl dipeptide), the presence of which would decrease the desired response. More specifically, the CTL inducing composition comprises the antigen to which the CTL response is desired and a non-toxic antigen formulation which comprises, consists or consists essentially of a stabilizing

20 detergent, a micelle-forming agent, and a biodegradable and biocompatible oil. This antigen formulation preferably lacks any immunostimulating peptide component, or has sufficiently low levels of such a component that the desired cellular response is not diminished. This formulation is preferably provided as a

stable microfluidized oil-in-water emulsion. That is, each of the various components are chosen such that the emulsion will remain in an emulsion state for a period of at least one month, and preferably for more than one year, without phase separation. The antigen and antigen formulation are mixed
5 together to form a mixture, and that mixture can be administered to the animal in an amount sufficient to induce CTL response in the animal.

By "non-toxic" is meant that little or no side effect of the antigen formulation is observed in the treated animal or human. Those of ordinary skill in the medical or veterinary arts will recognize that this term has a broad
10 meaning. For example, in a substantially healthy animal or human only slight toxicity may be tolerated, whereas in a human suffering from terminal disease (with a life expectancy of less than about three years) substantially more toxicity may be tolerated.

By "stabilizing detergent" is meant a detergent that allows the
15 components of the emulsion to remain as a stable emulsion. Such detergents include polysorbate 80 (TWEEN 80) (Sorbitan-mono-9-octadecenoate-poly(oxy)-1,2-ethanediyl; manufactured by ICI Americas, Wilmington, Del.), TWEEN 40, TWEEN 20, TWEEN 60, Zwittergent 3-12, TEEPOL HB7, and SPAN 85. These detergents are usually provided in an amount of approximately 0.05 to 0.5%,
20 preferably at about 0.2%.

By "micelle-forming agent" is meant an agent which is able to stabilize the emulsion formed with the other components such that a micelle-like structure is formed. Such agents preferably cause some irritation at the site of injection in

order to recruit macrophages to enhance the cellular response. Examples of such agents poloxamer 401 and include polymer surfactants described by BASF Wyandotte publications, e.g., Schmolka, *J. Am. Oil. Chem. Soc.*, 54:110 (1977) and Hunter et al., *J. Immunol.*, 129:1244 (1981), both hereby incorporated by
5 reference, PLURONIC L62LF, L101, and L64, L121, PEG1000, and TETRONIC 1501, 150R1, 701, 901, 1301, and 130R1. The chemical structures of such agents are well known in the art. Preferably, the agent is chosen to have a hydrophile-lipophile balance (HLB) of between 0 and 2, as defined by Hunter and Bennett, *Journal of Immunology*, 133:3167 (1984). The agent is preferably
10 provided in an amount between 0.001 and 10%, most preferably in an amount between 0.001 and 5%.

The oil is chosen to promote the retention of the antigen in oil-in-water emulsion, i.e., to provide a vehicle for the desired antigen, and preferably has a melting temperature of less than 65°C. such that emulsion is formed either at
15 room temperature (about 20°C. to 25°C.), or once the temperature of the emulsion is brought down to room temperature. Examples of such oils include squalene, Squalane, EICOSANE, tetratetracontane, glycerol, and peanut oil or other vegetable oils. The oil is preferably provided in an amount between 1 and 10%, most preferably between 2.5 and 5%. It is important that the oil is
20 biodegradable and biocompatible so that the body can break down the oil over time, and so that no adverse affects, such as granulomas, are evident upon use of the oil.

It is important in the above formulation that a peptide component, especially a muramyl dipeptide (MDP) be lacking. Such a peptide will interfere with induction of a CTL response if it provided in an amount greater than about 20 micrograms per normal human formulation administration. It is preferred that
5 such peptides are completely absent from the antigen formulation, despite their apparent stimulation of the humoral compartment of the immune system. That is, although such peptides may enhance the humoral response, they are disadvantageous when a cytotoxic T-lymphocyte response is desired.

The antigen formulation can be formed from only two of the above three
10 components and used with any desired antigen (which term includes proteins, polypeptides, and fragments thereof which are immunogenic), to induce a CTL response in the above animals or humans.

In preferred embodiments, the method consists essentially of a single administration of the mixture (antigen plus antigen formulation) to the human or
15 the animal; the human or animal is infected with a cancer or virus and suffers one or more symptoms (as generally defined by medical doctors in the relevant field) of infection from the cancer or virus; and the antigen formulation is non-toxic to the human or animal.

In other preferred embodiments, the antigen is chosen from melanocytic
20 differentiation antigens, for example: gp100 (Kawakami et al., *J. Immunol.* 154:3961-3968 (1995); Cox et al., *Science*, 264:716-719 (1994)), MART-1/Melan A (Kawakami et al., *J. Exp. Med.*, 180:347-352 (1994); Castelli et al., *J. Exp. Med.*, 181:363-368 (1995)), gp75 (TRP-1) (Wang et al., *J. Exp. Med.*,

186:1131-1140 (1996)), and Tyrosinase (Wolfel et al., *Eur. J. Immunol.*, 24:759-764 (1994); Topalian et al., *J. Exp. Med.*, 183:1965-1971 (1996)); melanoma proteoglycan (Hellstrom et al., *J. Immunol.*, 130:1467-1472 (1983); Ross et al., *Arch. Biochem Biophys.*, 225:370-383 (1983)); tumor-specific, 5 widely shared antigens, for example: antigens of MAGE family, for example, MAGE-1, 2, 3, 4, 6, and 12 (Van der Bruggen et al., *Science*, 254:1643-1647 (1991); Rogner et al., *Genomics*, 29:729-731 (1995)), antigens of BAGE family (Boel et al., *Immunity*, 2:167-175 (1995)), antigens of GAGE family, for example, GAGE-1,2 (Van den Eynde et al., *J. Exp. Med.*, 182:689-698 (1995)), 10 antigens of RAGE family, for example, RAGE-1 (Gaugler et al., *Immunogenetics*, 44:323-330 (1996)), N-acetylglucosaminyltransferase-V (Guilloux et al., *J. Exp. Med.*, 183:1173-1183 (1996)), and p15 (Robbins et al., *J. Immunol.*, 154:5944-5950 (1995)); tumor specific mutated antigens; mutated β -catenin (Robbins et al., *J. Exp. Med.*, 183:1185-1192 (1996)), mutated MUM-1 (Coulie 15 et al., *Proc. Natl. Acad. Sci. USA*, 92:7976-7980 (1995)), and mutated cyclin dependent kinases-4 (CDK4) (Wolfel et al., *Science*, 269:1281-1284 (1995)); mutated oncogene products: p21 ras (Fossum et al., *Int. J. Cancer*, 56:40-45 (1994)), BCR-abl (Bocchia et al., *Blood*, 85:2680-2684 (1995)), p53 (Theobald et al., *Proc. Natl. Acad. Sci. USA*, 92:11993-11997 (1995)), and p185 20 HER2/neu (Fisk et al., *J. Exp. Med.*, 181:2109-2117 (1995)); Peoples et al., *Proc. Natl. Acad. Sci., USA*, 92:432-436 (1995)); mutated epidermal growth factor receptor (EGFR) (Fujimoto et al., *Eur. J. Gynecol. Oncol.*, 16:40-47 (1995)); Harris et al., *Breast Cancer Res. Treat*, 29:1-2 (1994));

carcinoembryonic antigens (CEA) (Kwong et al., *J. Natl. Cancer Inst.*, 85:982-990 (1995)); carcinoma associated mutated mucins, for example, MUC-1 gene products (Jerome et al., *J. Immunol.*, 151:1654-1662 (1993), Ioannides et al., *J. Immunol.*, 151:3693-3703 (1993), Takahashi et al., *J. Immunol.*, 153:2102-2109 (1994)); EBNA gene products of EBV, for example, EBNA-1 gene product (Rickinson et al., *Cancer Surveys*, 13:53-80 (1992)); E7, E6 proteins of human papillomavirus (Ressing et al., *J. Immunol.*, 154:5934-5943 (1995)); prostate specific antigens (PSA) (Xue et al., *The Prostate*, 30:73-78 (1997)); prostate specific membrane antigen (PSMA) (Israeli, et al., *Cancer Res.*, 54:1807-1811 (1994)); PCTA-1 (Sue et al., *Proc. Natl. Acad. Sci. USA*, 93:7252-7257 (1996)); idiotypic epitopes or antigens, for example, immunoglobulin idiotypes or T cell receptor idiotypes, (Chen et al., *J. Immunol.*, 153:4775-4787 (1994); Syrengelas et al., *Nat. Med.*, 2:1038-1040 (1996)); antigens of HIV: gp160, gag, pol, nef, Tat and Rev; the malaria antigens: CS protein and Sporozoite surface protein 2; the Hepatitis B surface antigens: Pre-S1, Pre-S2, HBc Ag, and HBe Ag; the influenza viral antigens: HA, NP and NA; Hepatitis A surface antigens; Hepatitis C surface antigens; the Herpes virus antigens: HSV gB, HSV gD, HSV gH, HSV early protein product, human papillomavirus antigens, cytomegalovirus gB, cytomegalovirus gH and IE protein gp72; respiratory syncytial virus antigens: F protein, G protein and N protein.

The CTL inducing adjuvant can be combined with the agent which is capable of neutralizing, blocking, antagonizing or down regulating the activity of tumor secreted immunosuppressive factors and administered to the patient as a

single composition or the two components can be administered separately.

Administration can be achieved via numerous well known techniques. Such modes of administration include, for example, intradermal injection, subcutaneous injection, intraperitoneal injection, and intramuscular injection.

- 5 Furthermore, administration of agents capable of neutralizing or down regulating immunosuppressive molecules can be administered separately independent of adjuvant administration, for example intravenously or intraperitoneally. The preferred embodiment is to administer the antigen containing CTL inducing adjuvant formulation intradermally, intramuscularly or subcutaneously and the
- 10 neutralizing agent systemically via intravenous administration.

- Synergism should be observed in any disease condition where immunosuppressive factors such as $TGF\beta$ have an adverse effect on the host's ability in being able to elicit a therapeutic CTL response. Such diseases include by way of example many cancers and neoplastic growths, viral infections and
- 15 parasitic infections. Cancers which can be treated using the subject synergistic combination include, by way of example, breast cancer, brain cancer, cervical cancer, leukemia, lymphoma, prostate cancer, skin cancer, colon cancer, lung cancer, ovarian cancer, pancreatic cancer, liver cancer, bladder cancer, kidney cancer, myeloma, colorectal cancer, nasopharyngeal carcinoma and endometrial
- 20 cancer. Viral and parasitic infections treatable according the invention include, for example, papillomavirus, malaria, Hepatitis, Herpes, cytomegalovirus, respiratory syncytial virus and HIV. As discussed above, another important

aspect of the invention includes the induction of hematopoiesis. This is of significant therapeutic importance in, for example, cancer therapies.

In this regard, it is well known that cancer patients, especially at late stages of the disease, show suppressed hematopoietic activity due to suppression of stem or progenitor cells. This suppression is a result of factors such as radiation and chemotherapy which is used in cancer treatment as well as immunosuppressive factors which are secreted by tumors. Treatment with the inventive combination composition allows hematopoiesis to be restored or boosted. Moreover, it should further improve chemo or radio therapy as it should enable the therapeutic dosages to be administered without adverse effects.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

15

EXAMPLES

Example 1

Mice were inoculated with ovalbumin expressing EG7 cells (2×10^6 cells/mouse). Derivation of EG7 is described previously by Moore et al., *Cell*, 54:777 (1988). On day 7, post-inoculation mice bearing 250-350 mm³ size tumors were sorted in to 5 groups and treated as follows: Group A, the control group received no antigen injection (■), Group B received 30 μ g of ovalbumin in PROVAX s.c. (●), Group C received 30 μ g ovalbumin in PROVAX™ s.c. and 50

μ g of anti-TGF β antibodies i.p. per mouse (Δ), and Group D received 50 μ g of anti-TGF β antibodies i.p. (Δ). The data as set forth in Figure 1 indicates that the treatment of mice bearing progressively growing EG7 tumors with anti-TGF β antibodies in conjunction with ovalbumin in PROVAX™ gave enhanced anti-tumor activity under conditions where treatment with ovalbumin-PROVAX™ is not effective.

10 Example 2

Mice were inoculated with HPV-E7 expressing HOPE2 cells (4×10^6 cells/mouse) (2.A.). E7 expressing HOPE2 transfectant was obtained by electroporation of an E7 encoding mammalian expression plasmid into K1735-X21 cells (Kind gift from Dr. Isaiah J. Fidler). The Human Papillomavirus Type 16 E7 expression vector, INPEP4 + LE7, contains a 300 bp E7 encoding fragment (amino acid residues 2-97; Seedorf et al., *Virology*, 145:181-185 (1985)) fused downstream of an immunoglobulin leader sequence (L). Transcription is driven by the Cytomegalovirus promoter/enhancer (CMV) and the bovine growth hormone (BGH) 3' flanking sequence provides a polyadenylation signal for RNA processing. Bacterial neomycin phosphotransferase (N) and mammalian dihydrofolate reductase (DHFR) expression cassettes, driven by the mouse beta-globin major promoter (BETA), allow dominant selection by G418 and methotrexate, respectively. The

neomycin gene cassette includes the SV40 early polyadenylation signal (SV40) for RNA processing. Plasmid DNA is linearized by restriction digestion with PAC I prior to electroporation. K1735-X21 cells were grown in MEM Alpha medium (Gibco BRL.) supplemented to 10% (v/v) non-essential amino acids (Irvine Sci.), 10% (v/v) L-glutamine (Irvine Sci.), 20% (v/v) MEM Vitamin solution (Gibco BRL.), 1 mM Sodium Pyruvate (Biowhittaker), and 5% FBS (Gibco BRL.). 1 μ g of Pac I linearized INPEP4 + LE7 DNA was electroporated into 4×10^6 K1735-X21 cells and using a BTX 600 Electroporator (375 volts, 13 ohms, and 25 microfaradays). The cells were plated in a 96 well flat bottom plate. After 24 hours of incubation, the cells were fed by media supplemented with 0.4 mg/ml active G418. G418 resistant clones were screened for E7 expression by ELISA, Western and Northern blot analyses and selected for further expansion. HOPE2 was positive for E7 expression by all of the above analyses.

On day 11 post-inoculation, mice bearing 75-150 mm³ size tumors were sorted in to 4 groups and treated as follows: Group A, the control group received no antigen injection (\square), Group B received 30 μ g of E7 in PROVAX[™] s.c. (\diamond), Group C received 30 μ g ovalbumin in PROVAX[™] s.c. and 100 μ g of anti-TGF β antibodies i.p. per mouse (Δ) and Group D received single i.p. injection of 100 μ g of anti-TGF β antibodies (\circ). The data as set forth in Figure 2A indicates that the treatment of mice bearing progressively growing HOPE2 tumors with anti-TGF β antibodies in conjunction with E7-PROVAX[™] gave enhanced anti-tumor activity.

In another experiment, on day 13 post HOPE2 inoculation, mice were sorted and grouped as above. These groups of mice were treated similar to 2.A., except for Group C(Δ) and D(\circ), which received 4 injections of anti-TGF β antibodies every 4 days between day 15-29 (2.B.). The results are set forth in
5 Figure 2B.

While the invention has been described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material, combinations of material, and procedures selected for that purpose. Numerous
10 variations of such details can be implied and will be appreciated by those skilled in the art. Furthermore, all of the publications, patents and patent applications cited herein are incorporated by reference in their entirety.

Example 3

15 The concentration of TGF β 1 secreted by murine cell lines 3T3 (BALB/c origin), HOPE2 (C3H origin) EL4, and EG7 (C57BL/6) and human cell lines KB (epidermoid carcinoma ATCC # CCL-17) and A431 (epidermoid carcinoma, ATCC # CRL-1555) were measured by TGF β 1 ELISA kit (Genzyme Corp., Cat. # 80-3108). Figures 3A and 3B measure the TGF β 1 concentration from serum
20 free conditioned medium (CM) using GIBCO CHO-S SFM II (Cat. # 91-0456) after either 3 days (Cell Lines EL4 and EG7) or 5 days (KB, A431 and HOPE2) of continuous culture *in vitro* at 37°C. CM was centrifuged at 400xg for 5

minutes before analyzing for TGF β concentration as per manufactures instructions

Figure 3A measures the activity of CM directly (fully active TGF β 1) and after acid activation followed by neutralization according to manufacturers instructions (total TGF β 1). The fraction of latent TGF β 1 in CM was estimated by subtracting the active concentration of TGF β from the total TGF β concentration. As shown in Figure 3A all cell lines incubated *in vitro* secreted TGF β 1, and $\geq 98\%$ of the secreted material was in the latent form.

Figure 3B estimates the level of TGF β 1 in conditioned medium from the various cell lines after normalization for the total cell number present after the 2 or 5 days incubation at 37°C.

Example 4

Figure 4 demonstrates the binding activity of the anti-TGF β neutralizing antibody for either murine or human TGF β , after acid activation and neutralization according to manufactures instructions. Murine TGF β was obtained from BALB/c 3T3 conditioned medium (see Figure 3) and diluted with PBS to 0.2 ng/ml, and human TGF β was obtained from A431 CM and diluted with PBS to 0.4 ng/ml. Conditioned medium was incubated with various dilutions of monoclonal mouse anti-TGF- β 1, β 2, β 3 (Genzyme Corp: Cat. # 80-1835-03) for 3 hours at 4°C and assayed for unconjugated TGF β using the ELISA assay described in Figure 3. The anti-TGF β neutralizing antibody shows comparable binding to TGF β from both human and murine sources.

WHAT IS CLAIMED IS:

1. A composition comprising:
 - (a) an admixture comprising a cancer, viral or parasitic antigen expressed by said cancer, virally or parasitic infected cells and a microfluidized antigen formulation, said antigen formulation comprising:
 - (i) a stabilizing detergent,
 - (ii) a micelle-forming agent, and
 - (iii) a biodegradable and biocompatible oil,said antigen formulation being formulated as a stable oil-in-water emulsion; and
 - (b) at least one agent which is capable of neutralizing or down regulating the activity of immunosuppressive factors.
2. The composition of claim 1 wherein said antigen formulation consists essentially of said stabilizing detergent, micelle-forming agent, and biocompatible oil.
3. The composition of claim 1 wherein the detergent is selected from the group consisting of TWEEN 80, TWEEN 20, TWEEN 40, TWEEN 60, Zwittergent 3-12, TEEPOL HB7 and SPAN 85.
4. The composition of claim 1 wherein said detergent is provided in an amount ranging from approximately 0.05 to 0.5%.

5. The composition of claim 4 wherein the amount of detergent is about 0.2%.

6. The composition of claim 1 wherein said micelle-forming agent has
5 a hydrophile-lipophile balance of between 0 and 2.

7. The composition of claim 6 wherein the amount of said micelle-forming agent ranges from between 1.25 and 5%.

10 8. The composition of claim 1 wherein said micelle-forming agent is selected from the group consisting of poloxamer 401, PLURONIC L62Lf, PLURONIC L101, PLURONIC L64, PEG1000, TETRONIC 1501, TETRONIC 150R1, TETRONIC 701, TETRONIC 901, TETRONIC 1301 and TETRONIC 130R1.

15

9. The composition of claim 1 wherein the amount of said micelle-forming agent ranges from between 0.5 to 10%.

10. The composition of claim 1 wherein the oil exhibits an melting
20 temperature of less than 65°C.

11. The composition of claim 1 wherein the oil is selected from the group consisting of squalane, eicosane, tetratetracontane, pristane, and vegetable oils.

5 12. The composition of claim 1 wherein the amount of oil ranges from between 1 and 10%.

13. The composition of claim 12 wherein the amount of oil ranges from between 2.5 and 5%.

10

14. The composition of claim 1 wherein the detergent is polysorbate 80 and the micelle-forming agent is poloxamer 401.

15. The composition of claim 14 wherein the oil is squalane.

15

16. The composition of claim 1 wherein the detergent is selected from the group consisting of TWEEN 20, TWEEN 40, and TWEEN 80, the oil is selected from the group consisting of squalane, eicosane, olive oil and pristane and the micelle-forming agent is selected from the group consisting of poloxamer 401, and PLURONIC L62LF.

20

17. The composition of claim 1 wherein said immunosuppressive factors is TGF β .

18. The composition of claim 1 wherein said agent which is capable of neutralizing or down regulating the activity of tumor and host secreted immunosuppressive factors is an anti-TGF β antibody, a TGF β R-fusion protein, a TGF β analog, a TGF β binding protein or a TGF β R blocking antibody.

5

19. The composition of claim 1 wherein said agent which is capable of neutralizing or down regulating or preventing activation of tumor and host secreted immunosuppressive factors is a thrombospondin peptide or a TGF β R Fc-fusion protein.

10

20. The composition of claim 1 wherein said antigen formulation comprises squalane, TWEEN 80 and poloxamer 401.

21. The composition of claim 1 wherein said antigen is selected from the group consisting of gp100, MART-1/Melan A, gp75, tyrosinase, melanoma proteoglycan, MAGE, BAGE, GAGE, RAGE, N-acetylglucosaminyltransferase-V, mutated β -catenin, mutated MUM-1, mutated cyclin dependent kinases-4, p21 ras, BCR-abl, p53, p185 HER2/neu, mutated epidermal growth factor receptor, carcinoembryonic antigens, carcinoma associated mutated mucins, EBNA gene products, papillomavirus E7 protein, papillomavirus E6 protein, prostate specific antigens, prostate specific membrane antigen, PCTA-1, immunoglobulin idiotypes and T cell receptor idiotypes.

15

20

22. The composition of claim 1 wherein said composition is useful for treating cancer, viral or parasitic disorders.

23. In a method of treatment which includes the induction of a
5 cytotoxic T-lymphocyte response wherein the improvement comprises (i) the administration of an adjuvant which induces a cytotoxic T-lymphocyte response and (ii) the administration of an antagonist of an immunosuppressive factor; wherein the administration of adjuvant and antagonist is effected sequentially or concurrently, and in any order.

10

24. The method of claim 23 wherein said secreted immunosuppressive factor is TGF β .

25. The method of claim 24 wherein said adjuvant and antagonist are
15 administered sequentially.

26. The method of claim 25 wherein the CTL inducing adjuvant is administered intradermally, intramuscularly or subcutaneously and the TGF antagonist is administered intravenously.

20

27. The method of claim 23 wherein said treatment comprises treating diseases selected from the group consisting of neoplasms or cancer, parasitic infection and viral infection.

28. The method of claim 23 wherein said treatment comprises restoring or boosting hematopoiesis.

29. The method of claim 27 wherein said cancer comprises breast
5 cancer, brain cancer, cervical cancer, leukemia, lymphoma, prostate cancer, skin cancer, colon cancer, lung cancer, ovarian cancer, pancreatic cancer, liver cancer, bladder cancer, kidney cancer, myeloma, colorectal cancer or endometrial cancer.

10 30. The method of claim 27 wherein said viral infection comprises papillomavirus, Hepatitis, Herpes, cytomegalovirus, respiratory syncytial virus or HIV.

31. The method of claim 27 wherein said parasitic infection comprises
15 malaria.

32. A method of treating neoplastic or cancerous growths comprising administering to a patient in need thereof:

(a) an admixture comprising a cancer or tumor antigen expressed by
20 said cancer cells and a microfluidized antigen formulation, said antigen formulation comprising:

- (i) a stabilizing detergent,
- (ii) a micelle-forming agent, and
- (iii) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion;

5 wherein said admixture is administered to said patient in an amount sufficient to induce a cytotoxic T-lymphocyte response in said patient which is specific for the cancer or tumor antigen contained in said admixture, and

(b) a therapeutically effective amount of at least one agent which is capable of neutralizing or down regulating the activity of tumor and host

10 secreted immunosuppressive factors.

33. The method of claim 32 wherein said antigen is selected from the group consisting of gp100, MART-1/Melan A, gp75, tyrosinase, melanoma proteoglycan, MAGE, BAGE, GAGE, RAGE, N-acetylglucosaminyltransferase-V, mutated β -catenin, mutated MUM-1, mutated cyclin dependent kinases-4, p21

15 ras, BCR-abl, p53, p185 HER2/neu, mutated epidermal growth factor receptor, carcinoembryonic antigens, carcinoma associated mutated mucins, EBNA gene products, papillomavirus E7 protein, papillomavirus E6 protein, prostate specific antigens, prostate specific membrane antigen, PCTA-1, immunoglobulin

20 idiotypes and T cell receptor idiotypes.

34. A method of treating neoplastic or cancerous growths comprising administering to a patient in need thereof the composition of claim 1 in an amount sufficient to induce a cytotoxic T-lymphocyte response.

5 35. A method of restoring or boosting hematopoiesis comprising administering to a patient in need thereof:

(a) an admixture comprising a cancer, viral or parasitic antigen expressed by said cancer, virally or parasitic infected cells and a microfluidized antigen formulation, said antigen formulation comprising:

- 10 (i) a stabilizing detergent,
 (ii) a micelle-forming agent, and
 (iii) a biodegradable and biocompatible oil,

said antigen formulation being formulated as a stable oil-in-water emulsion; wherein said admixture is administered to said patient in an amount sufficient to
15 induce a cytotoxic T-lymphocyte response in said patient which is specific for the viral or cancer antigen contained in said admixture, and

(b) a therapeutically effective amount of at least one agent which is capable of neutralizing or down regulating the activity of tumor and host secreted immunosuppressive factors, wherein said admixture and said agent are
20 administered separately or in combination, and in any order.

36. The method of claim 34, wherein said antigen is selected from the group consisting of gp100, MART-1/Melan A, gp75, tyrosinase, melanoma

proteoglycan, MAGE, BAGE, GAGE, RAGE, N-acetylglucosaminyltransferase-V, mutated β -catenin, mutated MUM-1, mutated cyclin dependent kinases-4, p21 ras, BCR-abl, p53, p185 HER2/neu, mutated epidermal growth factor receptor, carcinoembryonic antigens, carcinoma associated mutated mucins, EBNA gene
5 products, papillomavirus E7 protein, papillomavirus E6 protein, prostate specific antigens, prostate specific membrane antigen, PCTA-1, immunoglobulin idiotypes or T cell receptor idiotypes.

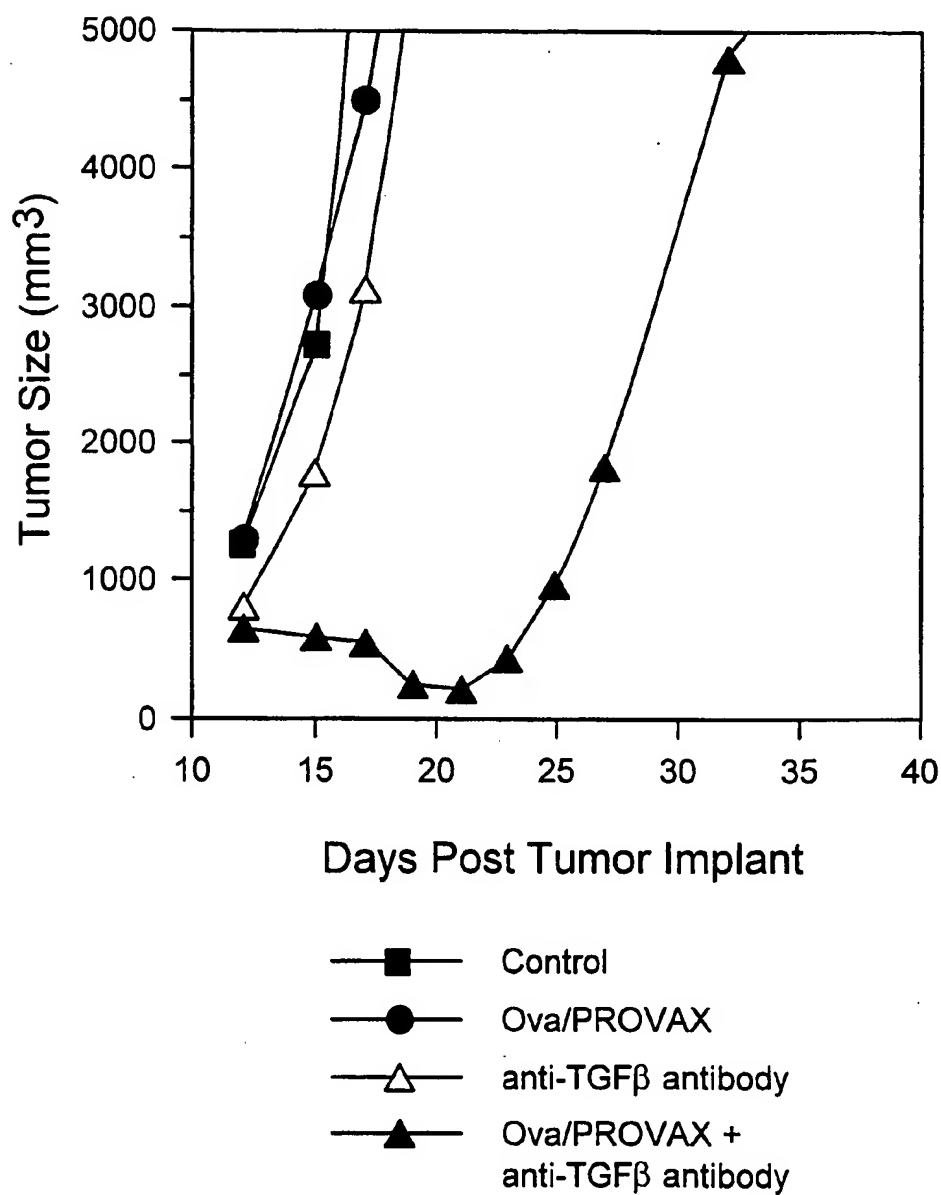
37. A composition comprising:

- 10 (a) an admixture comprising a cancer, viral or parasitic antigen expressed by said cancer, virally or parasitic infected cells and a microfluidized antigen formulation, said antigen formulation comprising:
- (i) a stabilizing detergent,
 - (ii) a micelle-forming agent, and
 - 15 (iii) a biodegradable and biocompatible oil,
- said antigen formulation being formulated as a stable oil-in-water emulsion; and
- (b) one or more TGF β antagonists.

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FIG. 1

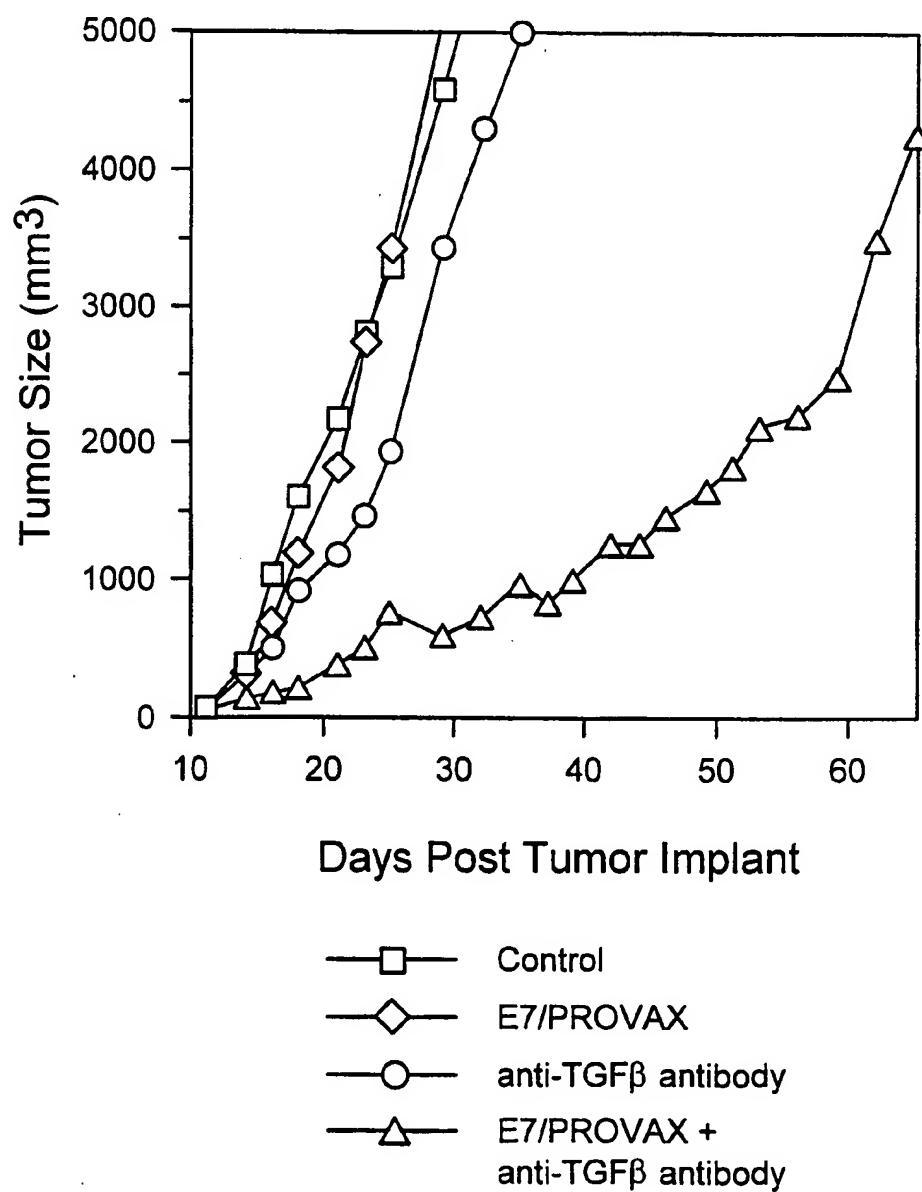
Anti-tumor activity of Ova-Provax + anti-TGF β antibody injection on established EG7 tumors



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FIG. 2A

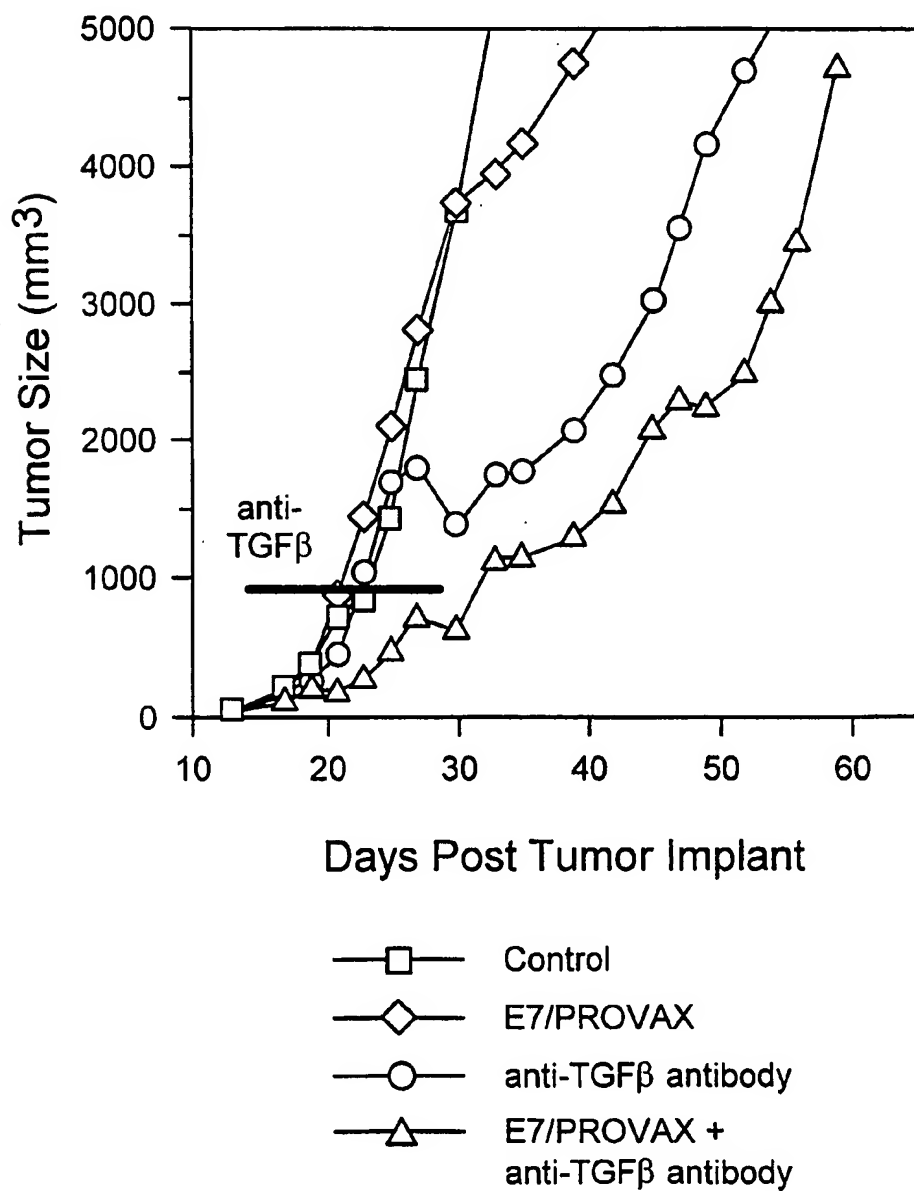
Anti-tumor activity of E7-Provax™ plus anti-TGFβ antibody injection on HOPE2 tumors



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FIG. 2B

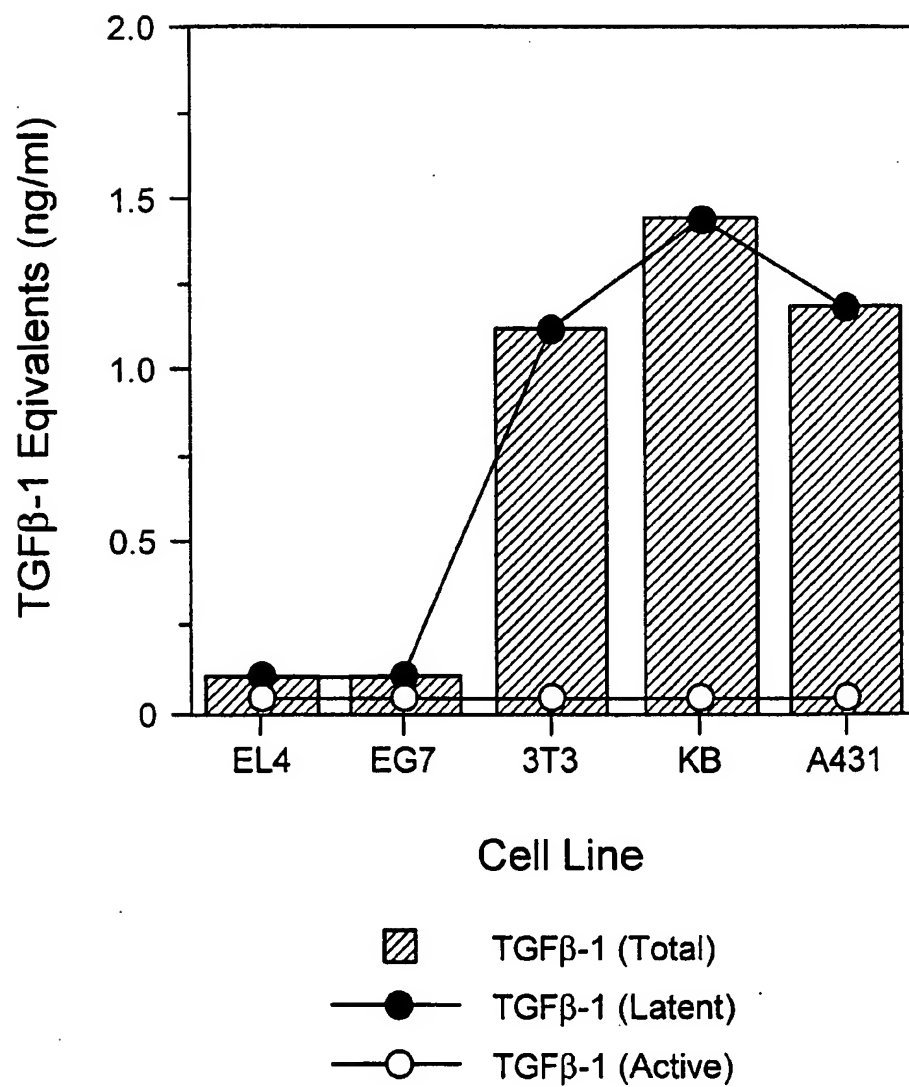
Anti-tumor activity of E7-ProvaxTM plus anti-TGFbeta antibody injection on HOPE2 tumors



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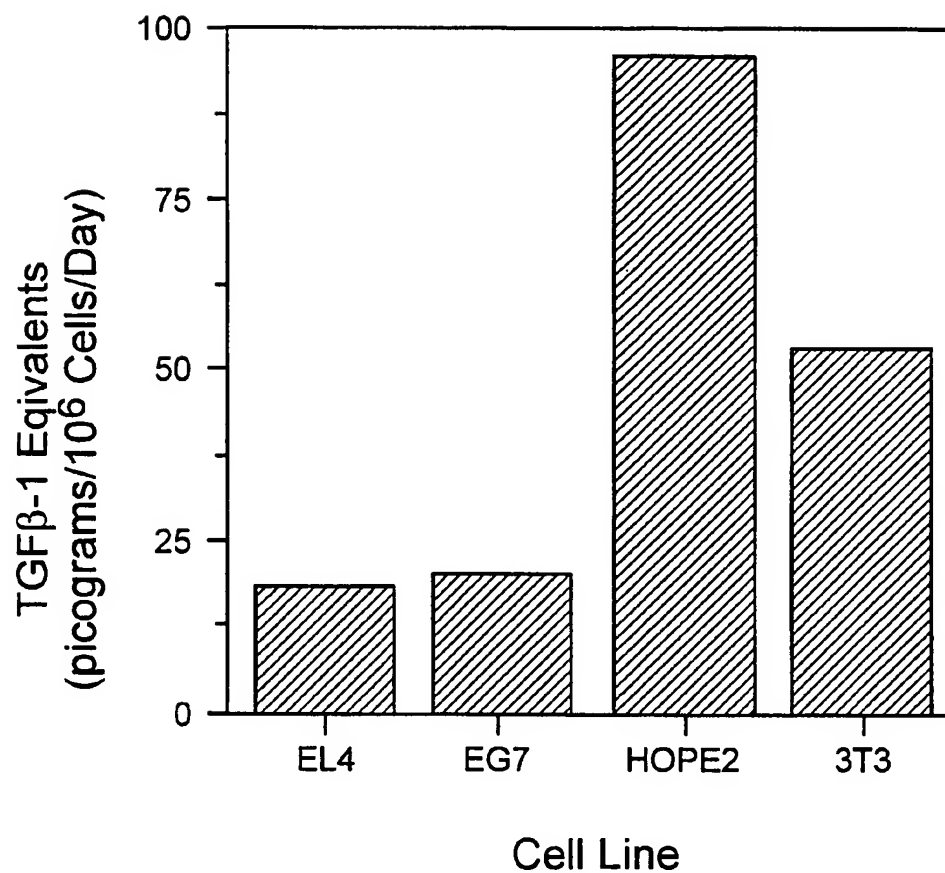
FIG. 3A

Measurement of TGF β 1 From Serum
Free Conditioned Medium by ELISA



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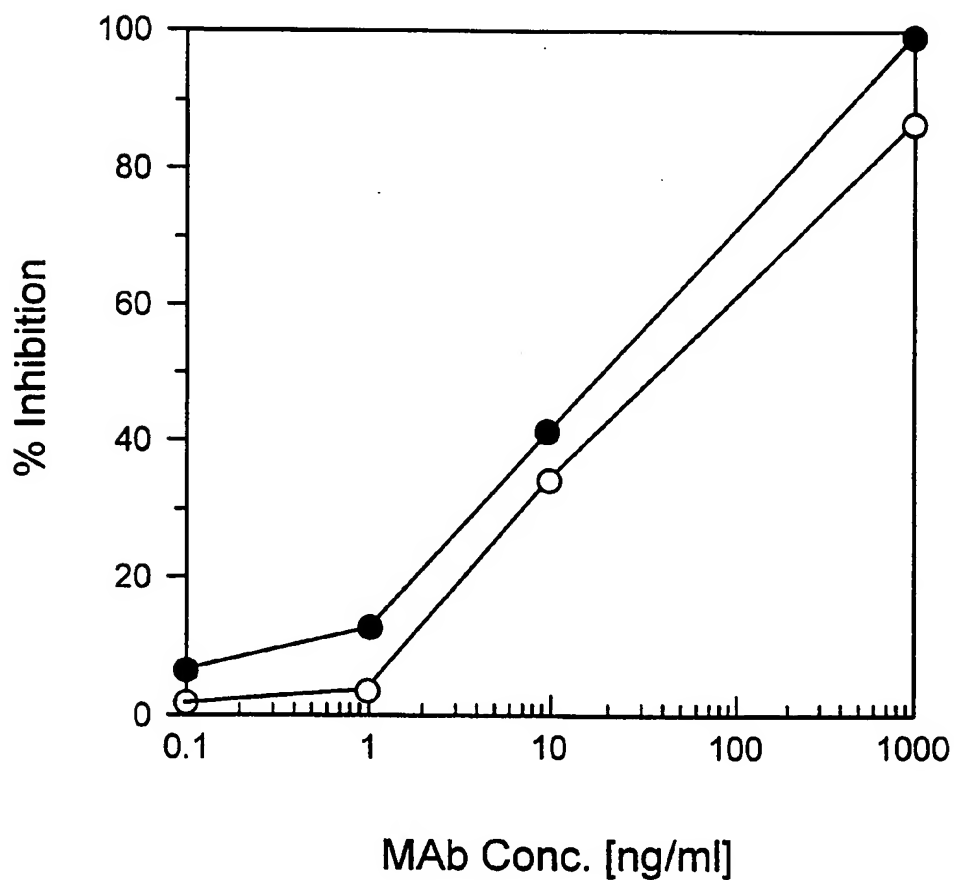
FIG. 3B



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FIG. 4

Binding Activity of Neutralizing TGF β MAb
for murine (Source: Balb/c 3T3 CM) or
human (Source: A431 CM) TGF β -1



Test: Competition ELISA, antigen:

—●— mTGF β (0.2 ng/ml/Rx)

—○— hTGF β (0.4 ng/ml/Rx)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18495

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 45/05, 39/12, 39/39, 9/107

US CL :424/184.1, 186.1, 278.1, 145.1, 204.1, 277.1, 283.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 186.1, 278.1, 145.1, 204.1, 277.1, 283.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,585,103 A (RAYCHAUDHURI et al) 17 December 1996, see the abstract; column 3, lines 57-67; column 4 and column 5.	1-37
Y	MURPHY et al., Differential Effects of Growth Hormone and Prolactin on Murine T Cell Development and Function. The Journal of Experimental Medicine, July 1993, Volume 178, pages 231-236, especially the upper left-hand column of page 232; the upper right-hand column of page 233; and the lower left-hand column of page 235.	1-37
Y, P	RICHARDS et al., Prolactin is an Antagonist of TGF-beta Activity and Promotes Proliferation of Murine B Cell Hybridomas. Cell Immunol., 15 March 1998, Volume 184, Number 2, pages 85-91, see the Abstract.	1-37



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

18 DECEMBER 1998

Date of mailing of the international search report

15 JAN 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18495

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS; DIALOG; file biochem

Search terms: TGF-beta, antigen, adjuvant, prolactin, immune response, superantigen, enhanc?, immunosuppress?,
micelle, detergent, oil